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Biology

PreImplantation Factor Reduces Graft-versus-Host Disease by Regulating Immune Response and Lowering Oxidative Stress (Murine Model)

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A B S T R A C T

Bone marrow transplantation (BMT) to treat severe hematologic malignancies often leads to potentially fatal acute graft-versus-host disease (GVHD), despite attempts at better donor–recipient matching and/or use of immunosuppressive agents. We report that embryo-derived PreImplantation Factor (PIF) plays a determining role in developing maternal/host tolerance toward the semiallogeneic or total allogeneic embryo and in regulating systemic immune response. Synthetic PIF treatment has proven effective in preventing immune attacks in nonpregnant models of autoimmunity. In this study, we tested the capability of PIF to prevent the development of acute GVHD in semiallogeneic or totally allogeneic murine BMT models. We examined the regulatory effect of PIF both in vivo and in vitro to control deleterious GVHD while maintaining its ability to preserve the beneficial graft-versus-leukemia (GVL) effect. Bone marrow and spleen cells from C57BL/6 donors were transplanted in semiallogeneic (C57BL/6xBALB/c) F1 or allogeneic (BALB/c) mice, which were then treated with PIF 1 mg/kg/day for 2 weeks. Short-term PIF administration reduced acute GVHD in both models and increased survival for up to 4 months after semiallogeneic or totally allogeneic BMT. This effect was coupled with decreased skin inflammation (semiallogeneic model) and decreased liver inflammation (both models), as well as reduced colon ulceration (allogeneic model). GVHD-associated cytokine and chemokine gene expression were decreased in the liver. PIF further lowered circulating *IL-17* levels, but not *IFN-γ* levels. Both in vivo and in vitro, PIF treatment was demonstrated to lead to decreased inducible nitric oxide synthase expression and decreased lipopolysaccharide-activated macrophages to lower nitric oxide secretion. Significantly, PIF did not diminish the beneficial GVL effect in the B cell leukemia model. PIF acts primarily by inducing the regulatory phenotype on monocytes/antigen-presenting cells, which controls T cell proliferation. Overall, our data demonstrate that PIF protects against semiallogeneic and allogeneic GVHD long term by reducing both target organ and systemic inflammation and by decreasing oxidative stress, while preserving the beneficial GVL effect.

INTRODUCTION

Allogenic bone marrow transplantation (BMT), performed in thousands of patients each year, is a well-established treatment modality for malignant and nonmalignant hematologic diseases. Within this type of stem cell graft, mature donor T cells are the main mediators of the beneficial induction of the beneficial graft-versus-leukemia (GVL) effect that prevents relapse of malignancy [1,2]. Unfortunately, these same cells also induce graft-versus-host disease (GVHD), a major cause of morbidity and mortality in BMT recipients [3]. Recent studies suggest the involvement of the inflammatory Th1 response as well as Th17 cells in autoimmunity [4,5].

Acute GVHD is characterized by widespread damage, affecting mainly target organs, such as the skin, liver, and gastrointestinal tract [6]. Protecting these vital organs and preventing the return of malignancy after immunosuppressive drug administration remain difficult clinical challenges.

Treating GVHD also poses serious challenges. Standard GVHD prophylaxis and therapy involve the use of drugs that cause generalized immune suppression, placing patients in danger of opportunistic infections and tumor relapse [2]. The initial management of acute GVHD usually includes steroids in combination with other immunosuppressive agents; however, this treatment is not effective in all patients [3]. Steroid-resistant GVHD may result in death [7]. Additional agents have been evaluated for treating and preventing GVHD [8], but none is currently in clinical use.

The ideal GVHD prophylaxis would exert 2 effects simultaneously: (1) prevent the grafted bone marrow cells from attacking their new host, and (2) sustain and maintain the new marrow's ability to destroy the host's remaining cancer

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cells. To obtain such a comprehensive effect, we turn to pregnancy, which we characterize as both a perfect immune regulatory environment and a successful transplantation model. The host/mother's immune system accepts a semi-allogeneic embryo (or a totally allogeneic one, in the case of a donor embryo) successfully. In pregnancies associated with infection or immune disorders, embryo tolerance continues to be preserved while maternal ability to fight disease is not only not impaired but may even become heightened, as needed. Pregnancy is an immune tolerant state, not an immunosuppressed state, in which the embryo/graft and mother/host synergize for best results. A number of mechanisms are involved in this feto-maternal tolerance. Some of these include modulated/regulatory immune cells, such as M2 macrophages and uterine natural killer (NK) cells [9,10]. Thus, the immunologic profile of pregnancy is compatible with that of the desired GVHD prophylaxis [11-14].

Our research found that PreImplantation Factor (PIF), an evolutionary conserved embryo-derived peptide (MVRIKPGSANKPSDD), is expressed by the embryo/fetus and placenta and present in the maternal circulation of viable pregnancies [15-20]. Our group demonstrated that synthetic PIF modulates decidual immunity [21,22], reduces NK cell toxicity, and promotes trophoblast invasion, which is required for successful fetal development [23]. It also has been demonstrated that PIF's global immune regulatory properties play an essential role in mediating pregnancy's unique immune milieu, allowing maintained defenses against pathogens and disease [20]. We reported that PIF orchestrates global anti-inflammatory effects in human peripheral blood mononuclear cells. PIF binds naive $CD14^+$ cells (monocytes/leukocytes), and in activated peripheral blood T cells and B cells, it blocks mixed lymphocyte reaction (MLR) proliferation and leads to a *Th2* cytokine bias while preserving the *Th1*-type response. This was evidenced by the effect of secretion and confirmed by gene expression [24]. We also reported that low-dose PIF is effective in reducing NK cell toxicity by reducing critical *CD69* expression [25].

In nonpregnant mice, we have demonstrated that PIF reverses paralysis and blocks spinal cord inflammation while promoting neural repair (chronic experimental autoimmune encephalomyelitis model) [26-28]. PIF also was shown to prevent autoimmune diabetes while restoring pancreatic function and insulin expression (nonobese diabetic model) [28]. In both models, PIF acts by reducing oxidative stress and inhibiting proinflammatory cytokine expression [26,28].

In the present study, we investigated the ability of PIF administration to prevent alloactivation and acute GVHD in a murine BMT model [29]. We examined PIF-induced protection by evaluating proinflammatory protein expression in GVHD-affected tissues, as well as relevant circulating cytokine levels. We assessed the effect of PIF on maintaining the important beneficial GVL effect. Finally, we identified the mechanisms involved in PIF-induced protection using cultured immune cells. We report that short-term, low-dose PIF protects against the development of acute GVHD while diminishing proinflammatory mediators. PIF-protective effects are exerted through monocytes, in which the reduced inducible nitric oxide synthase (*iNOS*) expression decreases nitric oxide (NO) secretion, thereby protecting against oxidative stress.

METHODS

Animals

Female 8- to 11-week-old C57BL/6, BALB/c, and (C57BL/6xBALB/c) F1 mice were obtained from Harlan Laboratories (Jerusalem, Israel). The study was conducted under appropriate conditions and was approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem in accordance with national laws and regulations for the protection of animals.

BMT

Recipient (C57BL/6xBALB/c) F1, BALB/c, or C57BL/6 mice received lethal whole-body irradiation with a single dose of 1000 rad (semiallogeneic and autologous) or 800 rad (allogeneic), and were reconstituted with 8×10^6 donor C57BL/6 bone marrow cells and 1.2×10^7 (semiallogeneic and autologous) or 2×10^6 (allogeneic) spleen cells administered to the tail vein on the following day. Bone marrow from donor mice was collected by flushing of the femur, humerus, and tibia into Dulbecco's PBS (Biological Industries, Kibutz Beit Haemek, Israel). Spleens were crushed through 70-μm screens into PBS. Bone marrow mononuclear cells and splenocytes were isolated after centrifugation on a Ficoll-Paque gradient. Percent BMT chimerism was measured in blood samples obtained 10 days after transplantation by fluorescent-activated cell sorting (FACS) analysis.

Peptide Synthesis

Synthetic PIF (MVRIKPGSANKPSDD) and scrambled PIF peptide (PIFscr) (GRVDPSNKSMPKDIA) as a control were obtained by solid-phase peptide synthesis (Peptide Synthesizer; Applied Biosystems, Foster City, CA) using 9-fluorenylmethoxycarbonyl chemistry. FITC-labeled PIF (FITC-PIF) and PIFscr (FITC-PIFscr) were produced by adding FITC on the N-terminus of PIF in the solid phase using L-alanine as a spacer. Final purification was performed by reversed-phase HPLC, and peptide identity was verified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry and amino acid analysis, purified to >95% by HPLC, and documented by mass spectrometry at Bio-Synthesis (Lewisville, TX).

PIF Administration

PIF or PIFscr was dissolved in PBS. Solvent peptide or PBS alone (as a control) was administered PIF (1 mg/kg/day) to the experimental groups on the day of BMT, using ALZET osmotic pumps (model 1002; Durect, Cupertino, CA), implanted s.c., slightly posterior to the scapulae. The pumps provided a continuous infusion (0.25 μL/h) for up to 14 days. Mice were followed up to 4 months without further therapy.

GVHD Evaluation

Mice were monitored daily for weight loss, diarrhea, ruffled skin, and survival, as described previously [29]. GVHD disease score, based on all of the foregoing factors (rated on a scale of 0-6), was calculated [6]. In addition, peripheral blood samples were collected from the tail vein at day 18 post-BMT.

Histological Analysis

Representative liver, skin, spleen, and colon samples were obtained from sacrificed mice and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 10-μm-thick sections, and stained with hematoxylin and eosin. The sections were examined under an optical microscope (CK40; Olympus, Tokyo, Japan), and images were captured with an Olympus DP50 camera. The histopathological score of the liver was based on the extent of leukocyte infiltration into the tissue. The histopathological score of the skin was determined by evaluation of epidermal interface changes, hair loss, and scar formation. The histopathological score of the spleen in the GVL model was based on the degree of leukemic cell infiltration to the tissue. The histopathological score of the colon was based on the degree of edema, crypt loss, crypt hyperplasia, and epithelial ulceration.

Serum Cytokine Evaluation

Mouse serum was obtained from peripheral blood. *IL-17* levels were measured by ELISA (Mouse *IL-17A* ELISA MAX; BioLegend, San Diego, CA). *IFN-γ* and *IL-4* levels were also measured by ELISA (eBioscience, Hatfield, UK).

RNA Expression Analysis

Liver and gut samples were obtained from mice at 45 days after semi-allogeneic, allogeneic, or autologous BMT and preserved in RNA Save (Biological Industries, Kibbutz Beit Haemek, Israel). RNA was extracted using the TRIzol reagent protocol (Invitrogen, Carlsbad, CA). DNA was removed from the samples using an RNeasy column and on-column treatment with RNase-free DNAse (Qiagen, Valencia, CA). cDNA was synthesized from 0.5 μg of total mouse liver RNA using the RT^2 First-Strand Kit (SA Biosciences, Frederick, MD), as reported previously [21]. Liver cDNA was applied to a 96-well mouse inflammatory response and autoimmunity microassay plate, and quantitative PCR (SA Biosciences) was performed using a DNA Engine Opticon

2 Thermocycler (Bio-Rad, Hercules, CA) with the following program: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 40 seconds, and 72°C for 30 seconds. Data analysis was performed using the SA Biosciences Web-Based PCR Array data analysis software.

GVL Effect Evaluation

After semiallogeneic BMT, F1 mice were inoculated with B cell leukemia/lymphoma 1 (BCL1) cells. Osmotic pumps dispensing PIF (1 mg/kg/day) or PBS (control) were implanted s.c. and left in place for 14 days. In the control group, F1 mice underwent transplantation with syngenic cells, followed by inoculation with BCL1 cells. For the survival assay, 2×10^4 BCL1 cells were used, and mice were followed up to day 16. At that point, the surviving mice were sacrificed, and DNA was extracted from their spleen cells and tested by PCR using BCL1 primers. In the splenomegaly assay, 1×10^4 BCL1 leukemia cells were used, and mice were followed for up to 18 days, with daily monitoring. On day 18 after BMT, the mice were sacrificed, their spleens were removed and weighed, and histological analysis was performed to confirm leukemic development.

In Vitro Proliferation Experiments

MLR was performed as described previously [30] using irradiated C57Bl/6 mouse splenocytes as stimulators and BALB/c mouse splenocytes as responder cells. PIF was added to the culture medium as indicated. For coculture experiments, monocytes were differentiated from whole bone marrow of C57Bl/6 mice in a medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) for 10 days as described previously [31], in the presence or absence of 200 nM PIF. Subsequently, 8×10^3 monocytes were cultured with 4×10^5 T cells for 4 days, activated by anti-CD3 antibody (BioLegend). H^3 thymidine was added to the culture during the last 18 hours of incubation. Cells were harvested onto glass-fiber filters, and H^3 thymidine incorporation was measured by liquid scintillation using a TopCount NXT analyzer (PerkinElmer, Waltham, MA).

Flow Cytometry Analysis

Bone marrow cells were collected by flushing of the femurs and tibias of C57Bl/6 mice. Blood cells were obtained from tails. Cells were incubated with 3.1 μM FITC-PIF (bone marrow) or 6.2 μM FITC-PIF (blood) or with the same concentrations of FITC-PIFscr conjugates (control) for 15 minutes before a 1-hour incubation with antibodies against markers for immune cell populations: anti-*CD3* PE (eBioscience), anti-*CD19* antigen-presenting cells (APCs; BD Biosciences, San Jose, CA) and anti-*CD11b* APCs (SouthernBiotech, Birmingham, AL). Chimerism was assessed using anti-H2d antibodies. *B7-H1* expression was tested on *CD11b* cells differentiated with GM-CSF in the medium for 10 days in the presence of 200 nM PIF in culture. *INF-γ* was added during in the last 24 hours, and anti-mouse *B7-H1* PE antibodies (eBioscience) were used for FACS analysis. Flow cytometry was performed with a MACSQuant analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany).

Nitrite Measurement and iNOS Quantitative PCR Analysis

RAW cells were cultured in DMEM medium for 4-5 days. PIF (200 nM) was added to the culture at day 0 and/or day 3 of the experiment. For cell activation, lipopolysaccharide (LPS) was added for the last 24 hours of the experiment. Nitrite accumulation, an indicator of NO production, was measured in cell culture supernatants using Griess reagent (a mixture at 1:1 of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 3% H_3PO_4; Sigma-Aldrich, St Louis, MO). The supernatants (100 μL) were mixed with an equal volume of Griess reagent, and absorbance at 550 nm was measured in a microplate reader. The nitrite concentration was calculated from an $NaNO_2$ standard curve.

Quantitative PCR

Detection of transcript levels of the *iNOS* gene in cDNA from PIF-treated RAW cells and PIF-treated tissues from mice that underwent allogeneic BMT (liver and colon) was performed using the TaqMan Gene Expression Assay Kit (Applied Biosystems). Mouse *HPRT-1* was used as a housekeeping gene transcript. Primers and probes were purchased from Syntezza Bioscience (Jerusalem, Israel). Data analysis was done using StepOne version 2.2 (Applied Biosystems). Each sample was analyzed in triplicate.

Statistical Analysis

GVHD and GVL data from the mouse experiments were analyzed using the Student *t* test or χ^2 test. Histological data were analyzed using the nonparametric Mann-Whitney *U* test. Gene expression and serum cytokine levels were analyzed using the Student *t* test. In vitro data were analyzed using the Student *t*, χ^2, or Mann-Whitney *U* test. Significance was set at $P < .05$.

RESULTS

PIF Protects against GVHD and Improves Survival after Semiallogeneic BMT

To test the prophylactic effect of PIF on GVHD, F1 (C57BL/6xBALB/c) mice underwent whole-body irradiation followed by semiallogeneic BMT from donor C57BL/6 mice. PIF (1 mg/kg/day) or PBS (control) was administered for 2 weeks via an s.c.-implanted continuous-release osmotic pump, starting on the day of BMT. GVHD disease scores (based on skin lesions, diarrhea, and body weight, on a scale of 0-6), were significantly lower in the PIF-treated mice than in PBS-treated controls at 4 and 6 weeks post-BMT (Figure 1A). The overall improved clinical condition of the PIF-treated mice was reflected by significant body weight recovery (Figure 1B) and improved survival (Figure 1C). All mice experienced weight loss after irradiation therapy and recovered at ~18 days post-BMT. In contrast to controls, the PIF-treated mice continued to gain weight, and 88% of them survived to the end of the experiment. PIF administration had no effect on mice chimerism, which was >96% (Figure S1).

To determine the specificity of PIF's activity, its anti-GVHD effect was compared in the same model using scrambled peptide (PIFscr) as control. PIFscr has the same amino acids as PIF but in a different order. Mice were monitored for 4 months after transplantation to determine differences in the effect of PIF and PIFscr on survival. In 2 independent experiments, mice receiving PIFscr (1 mg/kg/day for 2 weeks) exhibited no protective effects against acute GVHD. In comparison, those receiving PIF showed significant protection (Figure 1D and E). Notably, only 16% of the mice receiving PIFscr survived for more than 100 days post-BMT, compared with 91% of the PIF-treated mice. Furthermore, although PIF was administered for only 2 weeks, its protective effect lasted for more than 100 days post-BMT (Figure 1E). Our data confirm short-term PIF-induced long-term GVHD protection.

PIF Reduces Skin and Liver Inflammation

We examined the effect of PIF treatment on skin, liver, and colon histopathology. In our semiallogeneic model, GVHD affected mainly the skin and liver (Figure 2), and only occasionally the colon (data not shown). At 45 days post-BMT, mice were sacrificed, and biopsy specimens were obtained from the 3 types of tissues. PIF-treated mice had noticeably fewer and less-severe skin lesions (indicators of GVHD) compared with control mice (Figure 2A). Histological examination of skin biopsy specimens showed severe ulcerations and ruffled skin in control mice, but not in the mice receiving PIF. The differences in histopathological scores were similarly significant. Liver histology revealed lymphocytic infiltrates in control mice, indicating a severe inflammatory immune response, but not in PIF-treated mice (Figure 2B). The differences in histopathological scores were significant here as well. These data document PIF-induced protection of GVHD target organs.

PIF Down-Regulates GVHD-Associated Gene Expression in the Liver and Circulating IL17 Levels

The decreased lymphocyte infiltration to the liver in PIF-treated mice encouraged us to evaluate local inflammatory gene expression, specifically chemokines, cytokines, and their receptors. We used liver cDNA samples in a mouse inflammatory response and autoimmunity microassay, with analysis by quantitative PCR (qPCR). In PBS control-treated mice, proinflammatory gene expression increased by

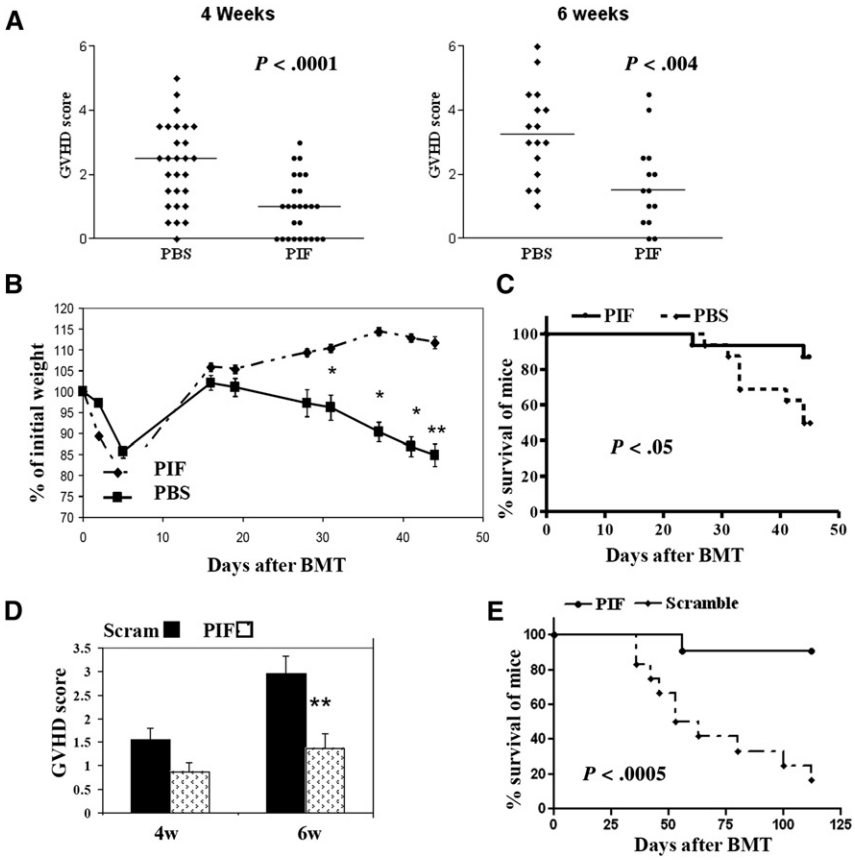

Figure 1. PIF reduces GVHD after semiallogeneic BMT. Mice underwent BMT with semiallogeneic bone marrow and spleen cells, and received PIF 1 mg/kg/day or PBS via s.c.-implanted osmotic pumps for 2 weeks. (A) GVHD scores at 4 and 6 weeks post-BMT; summary of 4 experiments. The difference between the PBS-treated control mice and the PIF-treated group is highly significant ($P \leq .0001$ at 4 weeks and $P \leq .004$ at 6 weeks, t test). The line represents median score of each group. (B) Weights from day 0 to day 45 post-BMT. The between-group difference is significant starting at day 27 ($P \leq .009$, t test). (C) Survival of mice from day 0 to day 45 post-BMT; summary of 2 experiments. Survival is significantly higher in the PIF-treated group compared with the PBS group ($^*P \leq .04$, χ^2 test). (D) GVHD score at 4 weeks and 6 weeks post-BMT; summary of 2 experiments. Mice underwent transplantation as described and were treated with PIF or PIFscr 1 mg/kg/day via s.c.-implanted osmotic pumps for 2 weeks. At 6 weeks, the difference between the PIFscr- and the PIF-treated groups is significant ($P \leq .004$, t test). (E) Survival of mice from day 0 to day 112 post-BMT; summary of 2 experiments. Survival is significantly higher in the PIF-treated group compared with the PIFscr group ($^{**}P \leq .0004$, χ^2 test).

>3-fold in 58% of the 84 genes tested, whereas in the PIF-treated mice, only 20% of the proinflammatory genes were elevated compared with naive mice (Figure 3A). Moreover, PIF treatment significantly prevented up-regulation of cytokines, chemokines, and associated receptors involved in liver GVHD (Figure 3B and C; details in Figure S2). Genes were considered relevant to GVHD if their expression was elevated in the semiallogeneic BMT liver but not in autologous BMT liver. Interestingly, among the genes associated with liver GVHD that were down-regulated by PIF, we found inducible nitric oxide synthase (*iNOS*). The increased expression of this gene in control mice liver was almost abolished in the PIF-treated mice (Figure 3D; details in Figure S2).

Several of the GVHD up-regulated genes in the liver were either Th1 or Th17 associated genes, immune pathways known to be activated in GVHD. These results are related mostly to infiltrating inflammatory cells in the liver. Thus, PIF's systemic effects were determined by evaluating circulating levels of representative *Th1*, *Th2*, and *Th17* type cytokines (*IFN-γ*, *IL-4*, and *IL-17*, respectively) in the serum of BMT recipient mice at 3 weeks post-transplantation. Interestingly, PIF-treated mice had markedly reduced serum levels of *IL-17*, but not of *IFN-γ* (Figure 3E). *IL-4* levels remained low in both groups (data not shown). The 2 groups are PIF-treated and PBS.

PIF Administration Does Not Interfere with the Beneficial GVL Effect

Based on our observation that PIF protects against the development of GVHD, we tested whether its effects interfere with the beneficial GVL effect normally associated with GVHD, using the BCL1 model [7]. This form of leukemia is characterized by extreme splenomegaly and leads to death in 100% of cases. We used spleen weight and survival to evaluate the effect of PIL on GVL activity. Lower spleen weight and increased survival would indicate a beneficial GVL effect.

We first tested PIF administration on the survival of post-BMT leukemic mice. Irradiated mice underwent semiallogeneic BMT as described above and were then inoculated with a large dosage of BCL1 cells (2×10^4). Two test groups were used: 1 group that received PIF 1 mg/kg/day for 2 weeks (BMT PIF group) and 1 group that received PBS for 2 weeks (BMT PBS group). A third group that underwent autologous BMT and was then inoculated with BCL1 cells served as a control group in which no GVL or GVHD effects were expected. Survival at day 16 was similar in the BMT PIF and BMT PBS groups, significantly different from that in the control group (Figure 4A). qPCR analysis on DNA extracted from the spleen of the surviving animals was performed to the ensure presence of cancerous cells in all mice.

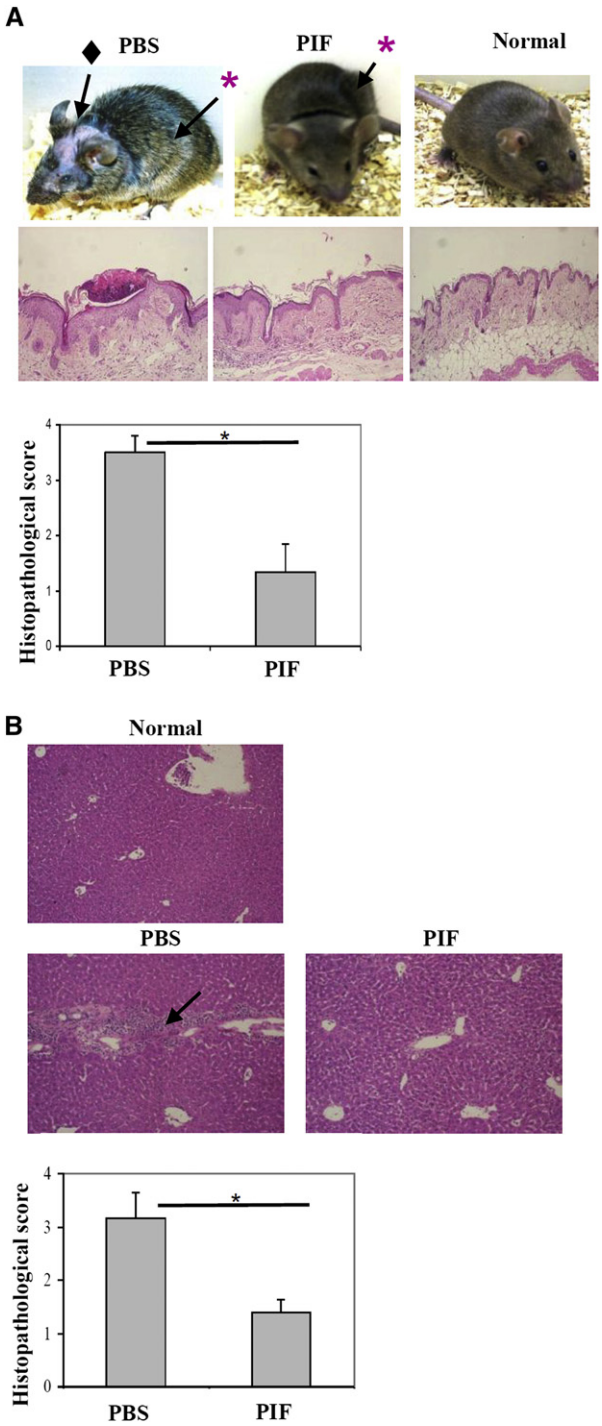

Figure 2. PIF reduces skin ulceration and liver inflammation. (A) The upper panels show representative pictures at 1 month post-BMT of a PBS-treated control mouse, a PIF-treated mouse, and a normal control (C57BL/6×BALB/c) F1 mouse. *, indicates osmotic pump; ◆, GVHD skin ulcers. The lower panels show representative histology of skin at 2 months post-BMT, along with average histopathological scores. The differences in histopathological scores are significant ($P \leq .02$, Mann-Whitney U test). (B) Representative liver histology at 2 months post-BMT and average histopathological scores. Lymphocyte infiltration is denoted by an arrow. The differences in histopathological scores are significant ($P \leq .03$, Mann-Whitney U test).

In the next set of experiments, new mice (divided into 3 groups, as above) with fewer BCL1 cells (1×10^4) were inoculated and sacrificed at 18 days post-BMT. Spleens were weighted and subjected to histological analysis. Spleen weights were markedly lower in both the PIF and PBS groups compared with the control group (Figure 4B), indicating the presence of a GVL effect. Histological analysis demonstrated the presence of leukemic cells in all mice, but the PIF and PBS groups had lower disease burden and better spleen tissue architecture compared with the control group (Figure 4C). These data indicate PIF-induced protection against GVHD coupled with the maintained GVL effect.

PIF is Equally Protective against GVHD in an Allogeneic BMT Model

Most of our results were obtained using a semiallogeneic BMT model (C57BL to C57BL/6xBALB/c F1). We also tested whether PIF equally protects against GVHD in a more severe model after full MHC mismatch, using a C57BL to BALB/c allogeneic murine BMT model. In this model, GVHD affected mostly the liver and colon. Similar to results for the semi-allogeneic model, we noted a significant reduction of GVHD with PIF treatment (Figure 5). In addition to improved disease scores (Figure 5A and B) and survival (Figure 5C), improved histopathological scores for liver (data not shown) and colon (Figure 5D) was evident. Histological analysis of the colon showed that PIF significantly reduced ulceration ($P = .02$; 4.7 degrees of freedom). Moreover, PIF reduced GVHD-induced *iNOS* expression both significantly in the liver (Figure 5E, right) and at borderline significance in the colon (Figure 5E, left).

PIF Regulates Immune Response Primarily through Monocytes

To gain insight into PIF's effects on the immune response, we examined whether PIF could also modulate allogeneic activation in vitro. To do so, we added PIF at various concentrations to an MLR of Balb/c and C57Bl mouse splenocytes, and found that PIF exerted a dose-dependent inhibitory effect on allogeneic proliferation (Figure 6A).

PIF is an embryo-derived peptide secreted at the embryo–maternal interface. In our model, it exhibits systemic effects; thus, we posited that PIF might regulate immune cell function. To establish which cells mediate the effects of PIF, we first used FITC-labeled PIF and PIFscr to identify associations with various immune cell populations. FITC-PIF avidly bound bone marrow–derived monocytes (*CD11b*$^+$ cells), but exhibited low binding to T cells (*CD2*) and B cells (CD19) (Figure 6B, upper). Similar results were obtained with circulating monocytes (Figure 6B, lower). Given that monocytes preferably interact with PIF, we examined the effect of *CD11b*$^+$ cells differentiated from bone marrow in the presence of PIF in a coculture with anti-*CD3* antibody–activated T cells. We found that PIF-pretreated monocytes significantly inhibited activated T cell proliferation (Figure 6C).

PIF Reduces iNOS Activity and Induces Regulatory Phenotype on Monocytes

Among the genes associated with liver GVHD that were down-regulated by PIF (Figure 3), we found elevated expression of the *iNOS* gene in control mice liver. The increase was almost abolished in PIF-treated mice (Figures 3D and 5D). PIF treatment was also associated with reduced *iNOS* expression in the colon (Figure 5C). Because *iNOS* can be synthesized by resident hepatic macrophages (Kupffer cells) in response to LPS and proinflammatory cytokines, as well as by other hepatic cells [32], we tested in vitro whether PIF would have the same inhibitory effect on

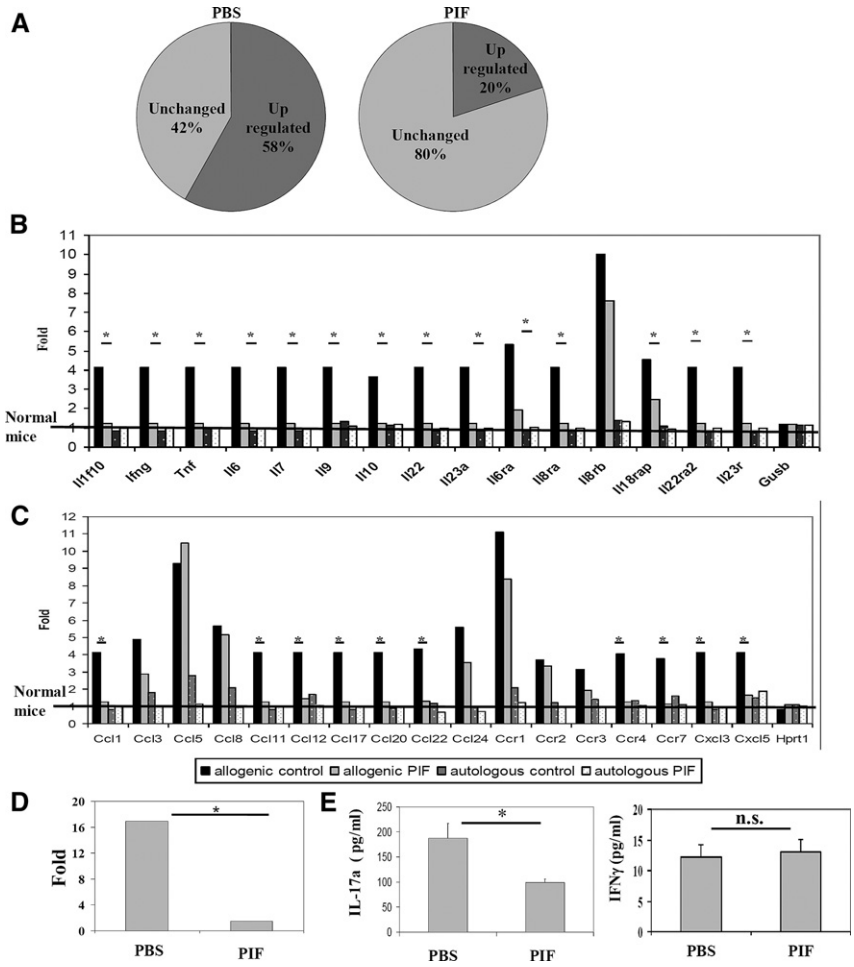

Figure 3. PIF down-regulates GVHD-associated gene expression in the liver. (A-D) qPCR analysis of inflammatory gene expression in the liver using a mouse inflammatory response and autoimmunity array. cDNA samples were obtained from livers of normal mice or mice after semiallogeneic/autologous BMT, treated with PIF or PBS as described in the text. n = 3 for each group. (A) Percentage of genes out of 84 inflammatory genes tested that were up-regulated at least 3-fold compared with their expression in normal mice. (B) Expression of cytokines and cytokine receptors involved in liver GVHD. *Gusb* is shown as a negative control. (C) Expression of chemokine and chemokine receptors involved in liver GVHD. *Hprt1* is shown as a negative control. (D) Expression of *iNOS* involved in liver GVHD. (E) Levels of proinflammatory cytokines in the serum of mice after semiallogeneic BMT, treated with PIF or PBS as in Figure 1. Blood samples were collected from tail veins at day 18 post-BMT. *IL-17* levels in the serum of PIF- and PBS-treated mice were measured by ELISA (*$P \leq .02$, *t* test), as were *IFN-γ* levels in the serum of PIF- and PBS-treated mice. The between-group difference is not significant.

a macrophage cell line. Indeed, after 3 days of PIF treatment, *iNOS* expression was reduced, and NO secretion was significantly reduced, in the LPS-activated RAW mouse macrophage cell line (Figure 6D and E).

B7H1 are regulatory receptors on the surface of monocytes. The expression of *B7-H1* receptors on host APCs plays a crucial role in GVHD regulation after BMT [33]. Our results show that PIF significantly up-regulates B7H1 receptor expression on bone marrow–derived monocytes (Figure 6F).

DISCUSSION

The BMT procedure to treat preexisting malignancy involves the destruction of cancer cells via a harsh conditioning procedure, that is, chemotherapy combined with specific and nonspecific immune responses of transplanted immune cells against the tumor, collectively known as the GVL effect [1,7]. Unfortunately, the nonspecific immune response that contributes to the desired GVL effect is also responsible for inducing deleterious GVHD [8]. To improve post-BMT prognosis, it is necessary to reduce GVHD without eliminating the GVL effect.

Pregnancy is a unique immune state, a "perfect transplant" in which there is no host-versus-graft (mother-versus-embryo) immune reaction, or, conversely, graft-versus-host (embryo-versus-mother) reaction, despite the semiallogeneity or total allogeneity of the fetus [9,14,34]. Moreover, pregnant women do not experience immune suppression, as evidenced by their maintained, adaptive, and strengthened as-needed ability to fight cancer and immune disorders (eg, leukemia, multiple sclerosis) [11,20]. We previously reported that PIF, an embryo-derived peptide, plays a determining role in creating this maternal immune tolerance toward the embryo, as well as promoting implantation [21,23]. In addition, PIF reverses paralysis while promoting neural repair and regeneration, and prevents the development of type 1 diabetes while preserving pancreatic insulin expression [26,28].

Herein we report that short-term, low-dose PIF administration prevents acute GVHD in both semiallogeneic and totally allogeneic BMT models, while maintaining the beneficial GVL effect. A low dose of PIF (1 mg/kg/day) administered for only 2 weeks increased long-term survival in a GVHD model and decreased GVHD-related symptoms

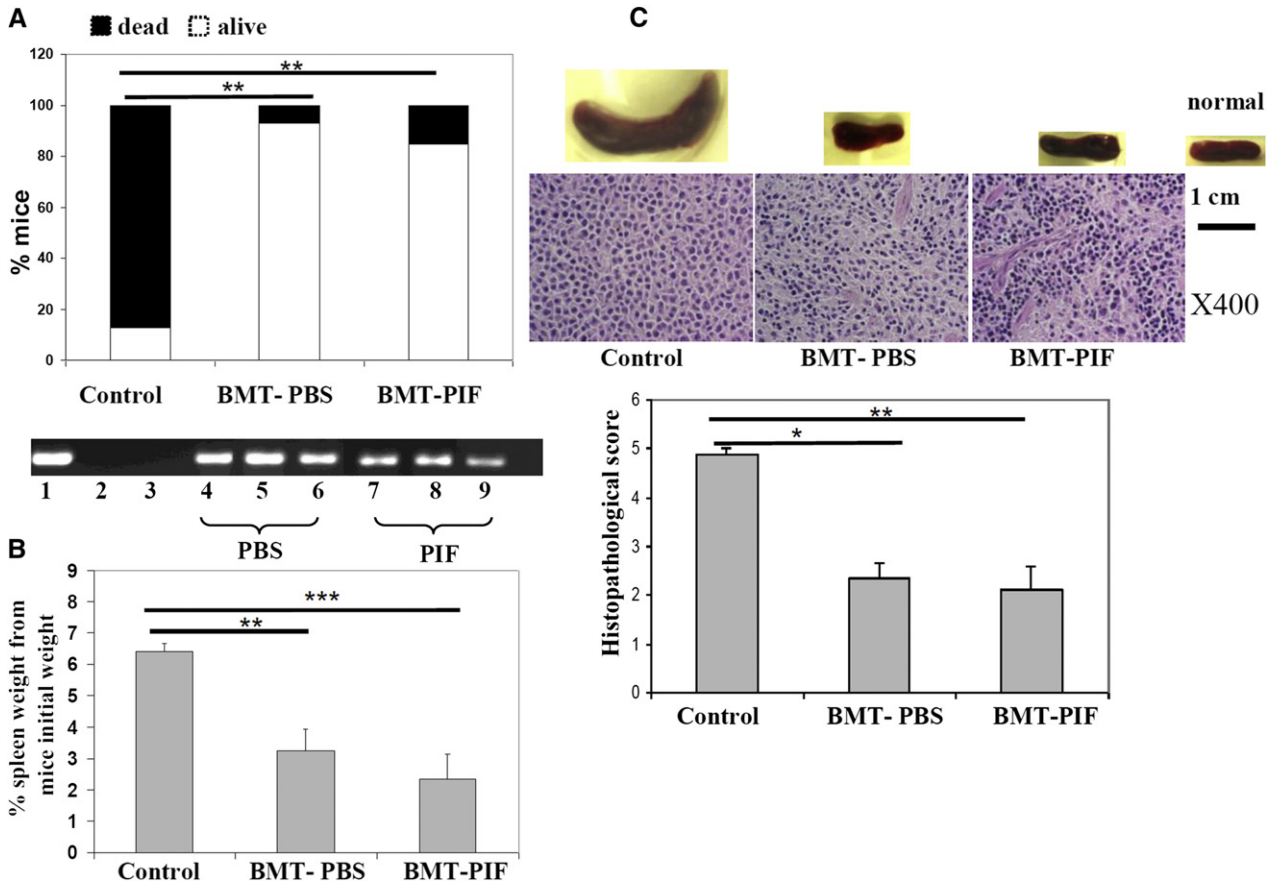

Figure 4. PIF does not interfere with the GVL effect. Mice were inoculated with BCL1 cells after semiallogeneic BMT and received PIF 1 mg/kg/day (PIF group) or PBS (PBS group) via s.c.-implanted osmotic pumps for 14 days. In the control group, mice underwent BMT with autologous cells and were then inoculated with BCL1 cells (BCL group). (A) Mouse survival at 16 days after BMT and inoculation of 2×10^4 BCL1 cells. The differences between the PBS and PIF groups and the BCL1 group are significant (PBS versus BCL1, $^{**}P \leq .001$; PIF versus BCL1, $^{**}P \leq .003$, χ^2 test). (B) Comparison of spleen size on day 18 after BMT and inoculation of 1×10^4 BCL1 cells. The differences between the PBS and PIF groups and the BCL1 group are significant (PBS versus BCL1, $^{**}P \leq .002$; PIF versus BCL1, $^{***}P \leq .0004$). (C) Histological analysis of the spleen on day 18 after BMT and inoculation of 1×10^4 BCL1 cells. The differences between the PBS and PIF groups and the BCL1 group are significant (PIF versus BCL1, $^{*}P \leq .002$; PBS versus BCL1, $^{**}P < .0001$, Mann-Whitney *U* test).

compared with PIFscr-treated controls for up to 100 days after cessation of therapy. The selected dose is in the same physiological range found in the serum of pregnant women. Our model uses semiallogeneic BMT (C57BL to C57BL/6 × BALB/c F1) [26]. We chose this model because in humans, transplantation from a haploidentical donor (parent to child) is the final option, and total mismatched donors are not yet used. GVHD is less severe in this model compared with our fully allogeneic model; however, long-term survival is poor in the semiallogeneic BMT model as well (Figure 1D). We have confirmed that PIF induced significant protective effects against a more severe form of GVHD using the fully allogeneic model (Figure 5). PIF's anti-GVHD effect is specific, and could not be replicated by PIFscr administration with a scrambled PIF peptide used as control.

PIF-treated mice maintained their weight and exhibited visibly less skin ulceration and inflammatory immune cell infiltration into the liver. In our semiallogeneic model, GVHD in the gut appeared only sporadically and thus was not examined further. In the allogeneic model, PIF reduced colon ulceration. In contrast, control mice experienced GVHD with expected weight loss, extensive skin ulceration, and liver inflammation. Overall, our data suggest that PIF offers measurable protection against damage in major clinical disease target organs, the skin, liver, and gut.

To provide mechanistic insight into the PIF-induced protection in the GVHD model, we performed several complementary analyses, including gene expression and circulating cytokine levels. We demonstrated that PIF blocks up-regulation of several proinflammatory genes in the livers of mice that underwent BMT, including cytokines, chemokines, and their receptors. Decreased expression of *IFN-γ*, *IL8r*, and *TNF-α* genes, which are associated with *Th1* response and inflammation, was documented. Although we did not directly assess *IL-17* expression in the liver, we found elevated expression of *IL-6* and *IL-23a*, which are important for *Th17* cell differentiation and proliferation. In addition, *IL-23R*, *CCR4*, *TNF-α*, *IL-22,* and *CCL20*, which are expressed by *Th17* cells, were increased. These findings imply involvement of *Th17* in liver GVHD [35]. PIF treatment prevented up-regulation of these genes, indicating elimination of the *Th17* response in the liver of mice that underwent BMT.

iNOS-induced NO secretion is a major element in oxidative stress-induced damage. Elimination of the marked elevation in *iNOS* in GVHD liver by PIF treatment supports the premise that PIF controls this critical pathway as well. Significantly, this protection was further confirmed in both liver and colon in the allogeneic BMT model, in which GVHD is more severe. PIF also reduced iNOS expression, leading to decreased NO secretion in cultured activated macrophages.

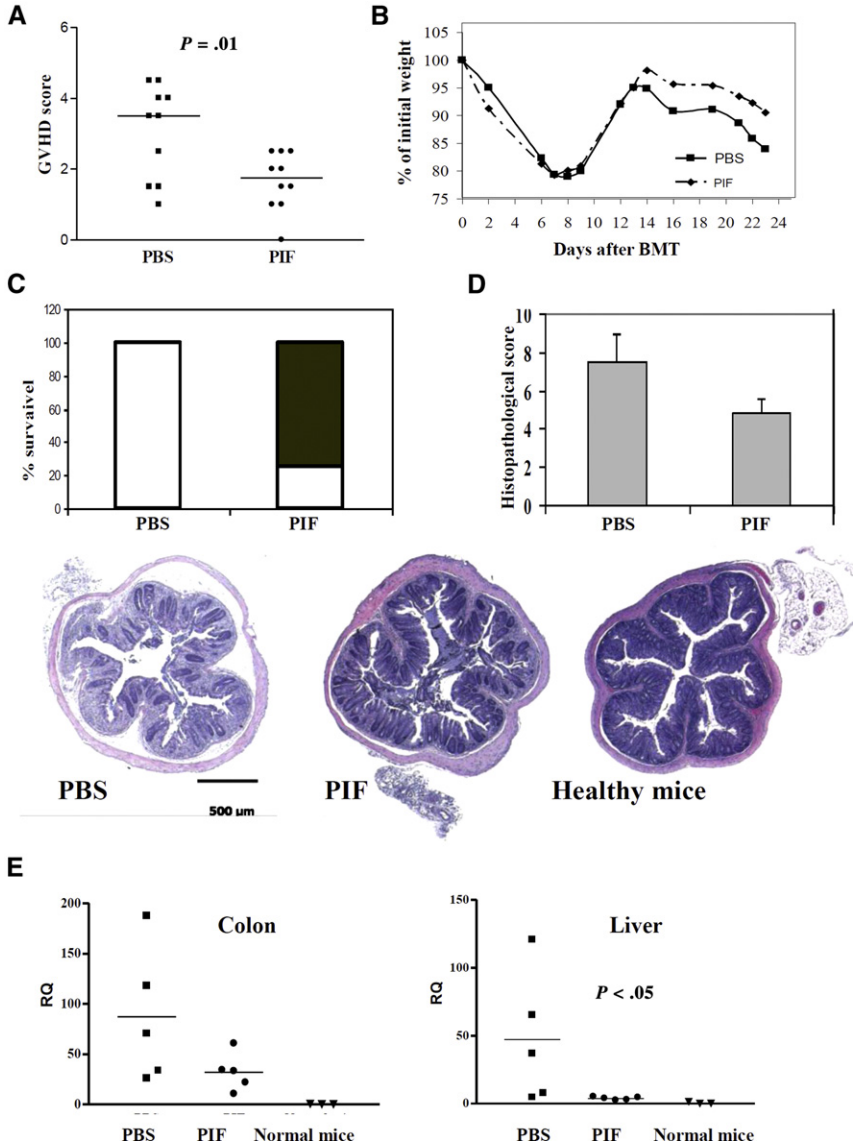

Figure 5. PIF protects against GVHD in the allogeneic BMT model. Mice underwent BMT with allogeneic bone marrow and spleen cells, and received PIF 1 mg/kg/day or PBS via s.c.-implanted osmotic pumps for 2 weeks. (A) GVHD score at 4 weeks post-BMT; summary of 2 experiments. The difference between the PBS-treated control mice and the PIF-treated group is significant ($P \leq .01$, t test). (B) Weight from day 0 to day 23 post-BMT. (C) Survival at 24 days post-BMT. Black indicates % live mice; white, % of dead mice. (D) Colon GVHD. The upper panel summarizes the results of histological analysis of the colon at day 23 post-BMT (n = 6/group). The lower panel presents representative histology pictures of PIF- and PBS-treated allogeneic BMT recipient mice and healthy mice. (E) qPCR analysis of *iNOS* mRNA expression in liver tissue (right) and in colon tissue (left) of PIF- and PBS-treated allogeneic BMT recipient mice and healthy mice. The between-group differences in liver expression are significant (PIF versus PBS, $P \leq .05$, Mann-Whitney U test).

Thus, a PIF-induced decrease in *iNOS* expression leads to reduced NO toxicity associated with liver and colon GVHD. Elevated *iNOS* expression in the GVHD liver has not been reported previously, which is not surprising given the fact that enhanced *iNOS* expression is closely related to the severity of disease in patients with autoimmune hepatitis [36] and inflammatory liver disease [37].

A role of NO in the intestinal pathology associated with murine GVHD has been reported previously [38]. The observation that PIF can decrease colon ulcers coupled with decreased *iNOS* expression supports PIF-induced protection of this target organ as well.

Taken together, our data imply that local regulation of organ inflammation play a critical role in PIF-induced protection. However, because in GVHD the inflammation is equally systemic, we examined circulating cytokine levels as well. PIF treatment reduced levels of circulating *IL-17*, a cytokine expressed primarily by *Th17* cells [4], but did not affect *IFN-γ* and the *Th2* cytokine *IL-4* levels. *Th17* cells direct the immune response against extracellular bacteria and fungi, but also contribute to autoimmune diseases [4], as well as GVHD [4,5,9,39]. The increased *IL-17* levels in murine GVHD serum and expression of *Th17*-related genes in the liver indicate a *Th17*-type inflammatory response [5]. The differences in *IFN-γ* expression in the liver and circulation might be related to differences in systemic effects versus local effects. PIF locally eliminated liver inflammation, but did not affect systemic *INF-γ*, thereby enabling a maintained *Th1* response that may be associated with GVL. Together with our in vitro results, these data suggest that PIF does not block the immune response, but rather gently modifies it by directing APCs to a more regulatory phenotype. This idea is in

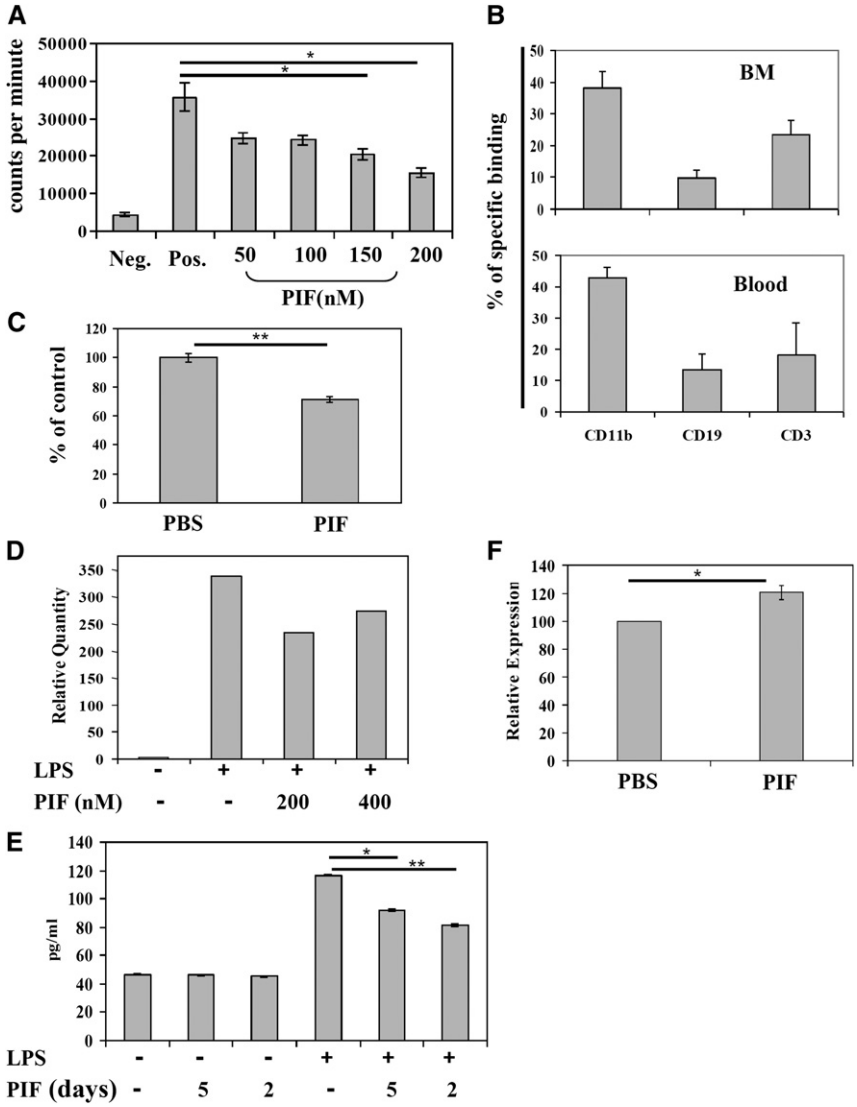

Figure 6. PIF has an immunomodulatory effect on monocytes in vitro. (A) Results of a thymidine proliferation assay of MLR using Balb/c and C57Bl mouse splenocytes in the presence of PIF. For negative control (Neg.), Balb/c mouse splenocytes were mixed with syngeneic irradiated splenocytes. One representative experiment is shown out of 3 experiments performed. The differences among the positive control (Pos.), 150 nM PIF, and 200 nM PIF are significant ($^*P \leq .03$, Mann-Whitney *U* test). (B) FACS analysis of bone marrow cells (upper panel) and blood cells (lower panel) stained with FITC-PIF or FITC-PIFscr and antibodies against CD3 (T cell marker), CD19 (B cell marker), and CD11b (marker for monocytes and granulocytes). The % of specific binding is the % of PIF-FITC binding cells minus the % of FITC-PIFscr (nonspecific) binding cells. Data are a summary of 2 experiments. (C) Thymidine proliferation assay of mouse T cells cocultured with bone marrow–derived monocytes; summary of 4 experiments. The monocytes were differentiated from bone marrow in the presence of 200 nM PIF, washed, and cocultured with T cells in the presence of anti-CD3 antibodies. The results are shown as % of control. The difference is highly significant ($^{**}P \leq .001$, Mann-Whitney *U* test). (D) qPCR analysis of *iNOS* mRNA expression in RAW cells untreated or treated with PIF for 3 days and activated with LPS for 24 hours. Mouse HPRT-1 was used as a control gene. (E) NO secretion from RAW cells untreated or treated with 200 nM PIF for the indicated times and activated with LPS for 24 hours. One representative experiment is shown out of 3 experiments performed ($^*P < .05$, $^{**}P < .05$). (F) *B7-H1* expression on bone marrow–derived monocytes, differentiated with GM-CSF in the medium for 10 days, in the presence of 200 nM PIF, by FACS analysis using anti-mouse *B7-H1* antibodies. *IFN-γ* was added in the last 24 hours of the culture. Data are a summary of 3 experiments ($^*P < .05$).

line with the observed differences in the effects of embryo-secreted PIF on the uterine milieu and systemic immunity during pregnancy [21,22,24,25]. The reduction in *IL-17* is also compatible with the PIF effect observed in the chronic experimental autoimmune encephalomyelitis model, in which spinal cord inflammation was reduced by inhibition of such a critical pathway [26].

Given that the majority of patients undergoing BMT have cancer, we examined whether PIF affects or interferes with the beneficial GVL effect. We report here that PIF does not impair the GVL immune response. Further studies are needed to examine this aspect of PIF-induced preserved GVL effect with different leukemia models to further substantiate this protective effect.

Our group recently reported that PIF orchestrates immune response in human immune cells [24,25]. The data reported herein confirm these observations, demonstrating that monocytes are the main target of PIF. These cells are part of the innate immunity first line of defense; they also serve as APCs, which direct the adaptive immune response. Evidence of PIF-induced regulation of monocytes can be characterized as follows: (1) PIF reduces allogeneic activation in an MLR test; (2) PIF-treated monocytes reduce CD3-activated T cell proliferation; (3) PIF increases expression of the regulatory receptor *B7H1* that binds CD28, a TCR co-receptor, which is crucial for GVHD regulation after BMT [33]; and (4) PIF reduces LPS-induced *iNOS* expression and NO secretion. Thus, the PIF-induced response can be exerted

through the regulation of APCs. Involvement of additional mechanisms is currently under investigation.

In conclusion, our findings indicate that short-term, low-dose synthetic PIF administration regulates the immune response, thereby preventing deleterious acute GVHD symptoms in the long term while preserving the beneficial GVL effect against cancer. The use of PIF in a clinical setting is warranted and is currently in late-phase planning.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.12.011.

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